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van Haastert, P.J.M.; Kuwayama, H

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Minireview

cGMP as second messenger during *Dictyostelium* chemotaxis

Peter J.M. van Haastert*, Hidekazu Kuwayama

Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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Abstract The chemoattractant cAMP induces directed cell locomotion in *Dictyostelium* cells. Several second messenger pathways are activated upon binding of cAMP to G-protein-coupled receptors, including adenylyl cyclase, guanylyl cyclase, phospholipase C, and the opening of plasma membrane Ca^{2+} channels. These second messenger responses are unaltered in many chemotactic mutants, except for the cGMP response. Activation of guanylyl cyclase depends on G-proteins and is regulated by a cGMP-binding protein in a complex manner. This cGMP-binding protein also mediates intracellular functions of cGMP to activate a PKC-related kinase that phosphorylates myosin II heavy chain, thereby allowing myosin filaments to rearrange during cell movement.

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Key words: Signal transduction; Osmotic stress; Guanylyl cyclase; Adaptation; Genetics

1. *Dictyostelium* development and chemotaxis

Dictyostelium is a fungus with distinct evolutionary traits. Comparisons of protein sequences suggests that it diverted about half-way between yeast and the splitting between animals and plants [1]. The amoeboid cells live in the soil where they feed on bacteria. Growing cells are chemotactically sensitive towards several compounds including folic acid and pterin, which are secreted by their prey [2]. When the bacteria become scarce, starvation induces the expression of a cAMP sensory system: some cells start to secrete cAMP to which cells in the surrounding respond with a chemotactic reaction. A cell aggregate is formed that can be composed of up to 100 000 cells. cAMP continues to be secreted by the cells inducing cell differentiation by complex mechanisms that are beginning to be understood [3–5]. Finally, a fruiting body is formed composed of highly vacuolized cells arranged in a stalk with on top spores embedded in a slime droplet. These spores are relatively safe to environmental stress; they resist extreme temperatures, humidity and pH, and they can pass the digestive track of birds and nematodes [6].

For chemotaxis at least two components are required: movement and orientation. Bacteria move by a combination of smooth swimming and tumbling. After a tumble, cells swim in a new random direction. Bacteria move in the direction of a chemotactic gradient, because tumbling is suppressed when an temporal increase of the chemoattractant is detected [7]. Thus, bacteria do not measure the spatial gradient, and they must move in order to read the gradient. In contrast, immobile *Dictyostelium* cells are still able to extend pseudopods in the direction of a cAMP gradient, as was shown using electro-

poreted cells in Ca^{2+} -buffered solutions [8]. Other experiments [9] suggest that cells do not show chemotaxis when placed in a steep spatial gradient of which the concentration is everywhere decreasing with time. It thus appears that cells use both spatial and temporal clues to orient in a cAMP chemotactic gradient. Detection of temporal clues is imposed by the biochemistry of guanylyl cyclase activation, which shows rapid and exact adaptation to constant stimuli (see below).

Directionality of movement is likely to be mediated by a differential occupancy of chemoattractant receptors between the front and back of the cell. Chemotaxis towards cAMP is very efficient: cells move at very low cAMP concentrations in shallow gradients with a 2% difference between the ends of a cell. It has been calculated that at threshold chemotactic responses only 400 surface cAMP receptors are occupied, about 5 more at the front than at the back of the cell (see [10]). Clearly, evolution has driven the mechanism of *Dictyostelium* chemotaxis to perform at the verge of stochastic and thermal fluctuations.

2. Chemoattractant receptors and G-proteins

Folic acid, pterin and cAMP are detected by surface receptors that interact with G-protein (see [11]). Four genes encoding the cAMP receptor have been identified, those for folic acid and pterin are unknown. The cAMP receptors are predicted to pass the membrane seven times, typical for receptors that interact with G-proteins. In the *Dictyostelium* genome, eight genes encoding $\text{G}\alpha$ and one $\text{G}\beta$ subunits have been identified [12,13]. Cells with a deletion of $\text{G}\beta$ can not chemotax to any chemoattractant. Deletion of $\text{G}\alpha$ -subunits demonstrate that $\text{G}\alpha 4$ interacts with folic acid and pterin receptors [14], while $\text{G}\alpha 2$ is essential for cAMP chemotaxis [15,16].

3. Chemotactic mutants

Although chemoattractants such as cAMP, folic acid and pterin are detected by different surface receptors and G-proteins, it is expected that somewhere in the transduction cascade to directed cell locomotion the signals merge into one pathway. It has been suggested that the same pool of guanylyl cyclase is activated by both cAMP and folic acid, because cells that are simultaneously sensitive to both chemoattractants do not show additivity of the cGMP response [17]. Based on these observations a screen for hunting chemotaxis mutants was devised. From about 10 000 mutagenized cells, 51 mutants were identified that do not chemotax to cAMP, 21 mutant do not chemotax to folic acid, and 10 mutants do not respond to either chemoattractant. These mutants, designated KI1–KI10, were investigated genetically and biochemically in detail [18]. One mutant (KI-3) appears to be the only false

*Corresponding author. Fax: (31) (50) 3634165.

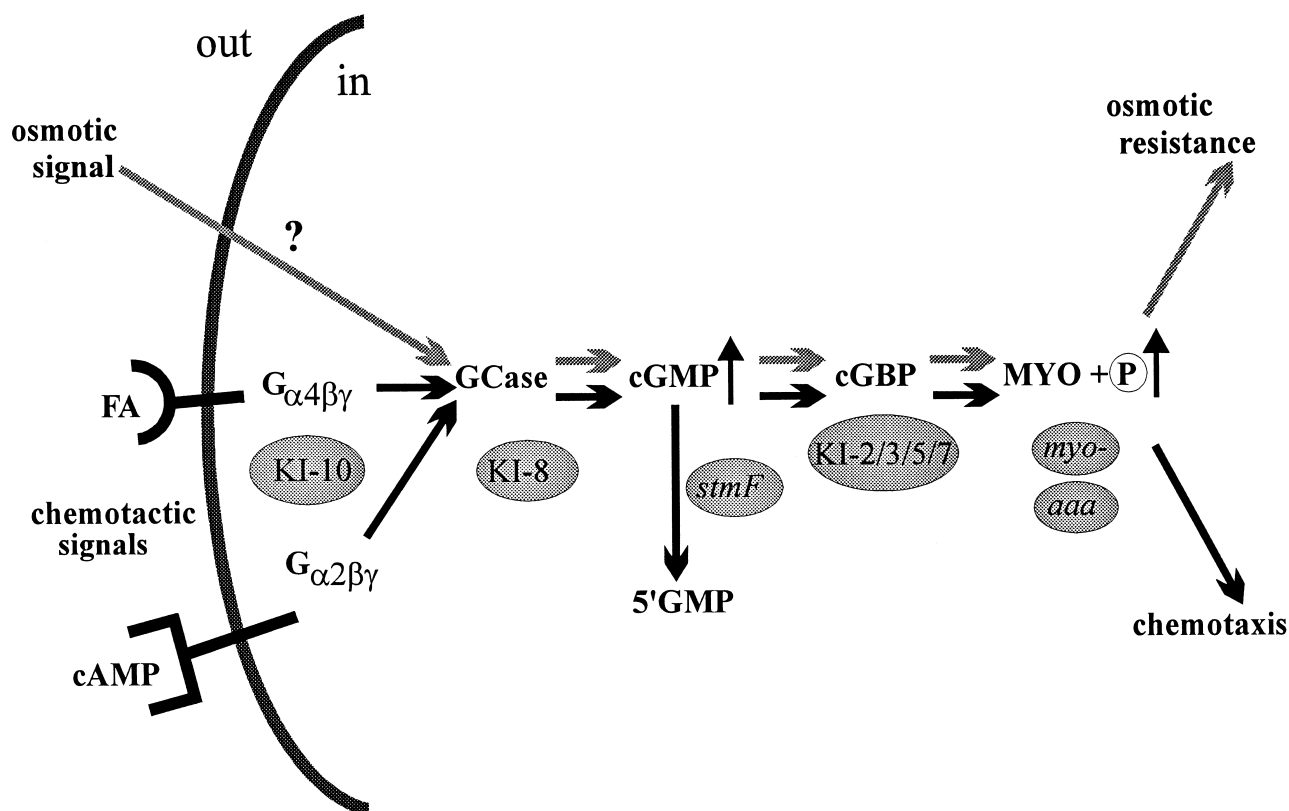


Fig. 1. Model of signal transduction of chemoattractants and osmotic stress via cGMP in *Dictyostelium*. Folic acid (FA) and cAMP bind to different surface receptors that interact with different G-proteins. These signals meet before guanylyl cyclase (GCase) producing the second messenger cGMP, that interacts with a cGMP-binding protein (cGBP) or is degraded by a cGMP-specific phosphodiesterase. This binding protein indirectly induces the phosphorylation of myosin II heavy chain (MYO); it also regulates guanylyl cyclase in a complex manner (not shown; see [33]). Guanylyl cyclase is also activated by osmotic stress via an unknown pathway that does not include cAMP receptors or G-proteins. The defect of KI and *stmF* mutants are discussed in the text. Besides guanylyl cyclase, chemoattractants activate several other second messenger pathways that are probably not involved in chemotaxis.

positive, all other mutant are severely defective, indicating the powerful selection method. Genetic analysis revealed that all mutants are recessive except KI-10. Interestingly this dominant mutant does not show chemotaxis to cAMP, folic acid and pterin, but responds normal to bacteria, yeast extract and human urine; this must mean that these complex mixtures contain still other unidentified chemoattractants. Further genetic analysis demonstrates that all mutants can complement each other [18]. Cloning the mutated genes should establish whether each complementation group belongs to a different gene.

Biochemical analysis demonstrate the normal activation of adenylyl cyclase and phospholipase C in the non-chemotaxis KI mutants, suggesting that these enzymes may not mediate chemotaxis [18]. This confirms the reversed experiments with mutants deleted in the genes encoding adenylyl cyclase [19] or phospholipase C [20] which show normal chemotaxis. KI mutants are severely defective in the activation of guanylyl cyclase or the detection of intracellular cGMP, suggesting that cGMP plays an essential role in mediating chemotaxis [18].

4. cGMP response in vivo

Chemoattractants induce a ten-fold increase of the intracellular cGMP concentration; maximal levels are obtained after 10 s and basal levels are recovered within 30 s. cGMP

levels start to rise after addition of cAMP with a lag-time of only 0.8 s, indicating that binding of cAMP to the receptor, and activation of the receptor, G-protein and guanylyl cyclase is completed within 1 s [21]. The cGMP response is controlled by an adaptation mechanism: when cells are restimulated at 30 s, they only respond to the second stimulus if its concentration is higher than that of the first stimulus. Adaptation is very fast with half-maximal adaptation after 2.5 s and complete adaptation after 10 s. When the second stimulus is applied later than 30 s, cells slowly regain responsiveness: de-adaptation shows first order kinetics with a half-time of about 90 s [22]. Both folic acid and cAMP induce similar cGMP responses; however, the responses are not additive, and cells stimulated with folic acid are adapted to folic acid, but not to cAMP and visa versa [23]. These observations suggest that folic acid and cAMP share the same pool of guanylyl cyclase, and that adaptation is localized in the unshared part of the transduction pathway, between receptors and guanylyl cyclase.

The kinetics of the cGMP response is extremely robust. Most drugs or mutations that interfere with signal transduction alter the magnitude of the cGMP response, but not the timing. There are two exceptions, mutant *stmF* and mutants KI-2 and KI-7. The cGMP response in *stmF* is both enhanced and prolonged, due to a mutation in the structural gene of a specific cGMP phosphodiesterase [24–26]. In wild-type cells

the enzyme is activated about 3-fold by cGMP. Detailed non-equilibrium kinetics suggests that in vivo this activation is mainly responsible for the rapid reduction of cGMP levels after 10 s [27,28]. Although mutants KI-2 and 7 also show a delayed cGMP response, cGMP phosphodiesterase activity is normal [18]. These mutants appear to be defective in the inhibition of guanylyl cyclase by a cGMP-binding protein (see below), resulting in prolonged guanylyl cyclase activity.

5. Regulation of guanylyl cyclase

Dictyostelium guanylyl cyclase is strongly inhibited by Ca^{2+} ions; inhibition is cooperative with a Hill coefficient of 2.3; half-maximal inhibition occurs at about 50 nM Ca^{2+} [29]. Experiments with permeabilized cells and computer simulations suggest that Ca^{2+} regulates the magnitude of the cGMP response, not its timing [28]. The inhibition of *Dictyostelium* guanylyl cyclase activity by Ca^{2+} is similar to the regulation of this enzyme in the vertebrate eye [30].

Mutant KI-8 has a strongly reduced guanylyl cyclase activity. Mutant KI-10 has normal basal guanylyl cyclase activity which, however, can not be stimulated by cAMP or folic acid. Mutants KI-2 and KI-7 show a delayed cGMP response with a maximum at 20 s after stimulation versus 10 s in wild-type cells. Mutants KI-4, 5, 6, and 9 do show a cAMP-mediated cGMP response, but only at elevated stimulus concentrations [18].

In cell lysates Mg^{2+} -dependent guanylyl cyclase activity is membrane bound, but requires a protein from the cytosol to show full activity [31]. Furthermore, guanylyl cyclase activity is strongly reduced at conditions where protein kinase activity is high [32]. Recent observations [33] show that the cytosolic factor is absent or non-functional in mutants KI-4 and KI-5, whereas inhibition of guanylyl cyclase activity by phosphorylation reactions is no longer detected in mutants KI-2 and KI-7. These mutants have a defect in a cytosolic cGMP-binding protein [34]. Thus, we have proposed that this cGMP-binding protein is a kinase that regulates guanylyl cyclase activity [33].

6. Function of intracellular cGMP

The intracellular protein that binds cGMP has been characterized to some extent. This protein is unaltered in *stmF* mutants, demonstrating that it is not (part of) a cGMP-phosphodiesterase [25], but most likely a cGMP-dependent protein kinase [35]. Kinetic studies of cGMP-binding to the protein reveal two binding forms: cGMP may dissociate fast (F-form) or slow (S-form). Oligonucleotides promote the S-form [36]. In KI-4/5 mutants the cGMP-binding sites are locked in the S-form whereas in KI-2/7 only the F-form can be detected; oligonucleotides have no effect on cGMP-binding in these mutants [34]. It is not clear whether these mutants are defective in the structural gene of the cGMP-binding protein/kinase.

Several experiments suggest a connection between cGMP and myosin heavy chain phosphorylation [37,38] by a PKC homolog that is activated indirectly by cGMP [39]. cGMP also plays a role in regulating the phosphorylation of myosin light chain [40]. Phosphorylation of myosin heavy and light chains is promoted in mutant *stmF* with no cGMP phosphodiesterase, whereas it is absent in mutants KI-10 and KI-8

with no cGMP response, or in mutant KI-4 with an altered intracellular cGMP-binding protein ([38,40], Kuwayama and Van Haastert, unpublished observations). It is unlikely that phosphorylation of myosin is the only target of intracellular cGMP. Cells in which the myosin heavy chain is deleted display nearly normal chemotaxis [41], whereas mutants defective in cGMP synthesis show no chemotaxis.

7. cGMP and osmo-regulation

Cells have two main strategies to cope with hyper-osmotic stress: using a wall to provide resistance against the turgor pressure, or adjusting the intracellular osmotic value to that of the outside. *Dictyostelium* has still another strategy, which is to rearrange actin and myosin filaments under the cortex. This may provide physical resistance to osmotic stress. Intracellular cGMP plays an essential role in this process.

In wild-type cells, osmotic stress induces the synthesis of large amounts of cGMP [42], which in turn leads to myosin phosphorylation and redistribution of myosin filaments [43]. Cells lacking myosin heavy chain or guanylyl cyclase are more sensitive to osmotic stress than wild-type cells. Mutant cells are also osmo-sensitive when myosin can not be phosphorylated, either by mutating the phosphorylated threonines to alanines [43], or in mutants KI-4 and KI-5 where the intracellular rise in cGMP can not be transmitted to the myosin kinase. On the other hand, chemotaxis mutants *car1⁻/car3⁻*, *gβ⁻* and KI-10 with mutation upstream of guanylyl cyclase are osmo-resistant and show a good osmo-induced cGMP response, suggesting that the osmo-resistance and chemotaxis pathway use different detectors, but share the cGMP machinery (Fig. 1; Kuwayama and Van Haastert, unpublished observations).

8. Conclusions

Mutant analysis clearly demonstrate that the second messenger cGMP plays a pronounced role in chemotaxis. Intracellular cAMP may not be important at all, whereas the function of PLC-derived second messengers ($\text{Ins}(1,4,5)\text{P}_3$, DAG and Ca^{2+}) is not clear. Identification of the genes encoding proteins producing, degrading or detecting cGMP should allow to unravel the early steps in the transduction of chemotactic signals from receptor to second messengers. This should then be connected with the wealth of information on the regulation of the locomotion apparatus that is rapidly emerging. Indeed, *Dictyostelium* may be the first eukaryotic system where chemotaxis is understood at a molecular level.

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